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**TITLE:** Production of Motif-Specific and Context-  
Independent Antibodies Using Peptide  
Libraries as Antigens

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**PRODUCTION OF MOTIF-SPECIFIC AND CONTEXT-  
INDEPENDENT ANTIBODIES USING PEPTIDE LIBRARIES AS  
ANTIGENS**

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**BACKGROUND OF THE INVENTION**

The present invention relates to the production of motif-specific, context-independent antibodies which are specific to at least one fixed amino acid residue in the context of variable surrounding amino acid or peptide sequences. Antibodies with these properties are useful in characterizing various forms of cellular regulation as well as serving to profile genome wide changes in cellular protein levels and protein modification.

Identifying the targets of intracellular signaling cascades are of major importance in understanding cell growth, differentiation, and cell death. Protein kinase cascades relay information from the cell surface to multiple cellular compartments including the nucleus and more distant cell processes such as synapses (Karin et al., *Curr. Opin. Cell. Biol.* 6:415-424 (1994)). Although a few targets of protein phosphorylation have been identified, most remain unknown, particularly those that regulate cell growth and differentiation. For example, the MAP kinase cascade is known to play an important role in the regulation of cell growth (Lewis et al., *Adv. Cancer Res.* 74:49-139 (1998), Crowley et al., *Cell* 77:841-852 (1994)). However, beyond a handful of substrates, few protein

targets responsible for the diverse actions of the MAP kinase cascade have been identified (Fukunaga and Hunter, *EMBO* 16(8):1921-1933 (1997), Stukenberg et al., *Curr. Biol.* 7:338-348 (1997)).

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Another example of cell signaling proteins are the 14-3-3 proteins, which represent a phylogenetically conserved family of phosphoserine binding proteins whose precise role in cell signaling has yet to be determined (Burbelo and Hall, *Curr. Biol.* 10 5(2):95-96 (1995)). These proteins represent a large fraction of total brain protein and are known to bind a wide variety of signaling molecules including: ras, raf, bad, cdc25, and many others (Yaffe et al., *Cell* 91:961-971 (1997)). Recently, it has been shown that 14-3-3 proteins bind specifically to 15 phosphorylated sites on proteins with the following motif: RXRSXS\*XP where S\* is phosphoserine and X represents any amino acid (Muslin et al., *Cell* 84:889-897 (1996), Yaffe et al. *supra*(1997)).

20 Similarly, histones have long been known to be modified by acetylation at specific lysine residues. Acetylation of lysine in histones is thought to reduce protein-DNA interactions and serve to open chromatin in regions undergoing transcription (Struhl, *Genes & Development*, 12:599-606 (1998)). Recently, 25 other proteins associated with transcription complexes have been shown to be acetylated on lysine although the functional

significance is unclear (Imhof et al., *Curr. Biol.* 7:689-692 (1997), Struhl *supra* (1998)).

Antibodies against phosphotyrosine have proven to be of  
5 great value in identifying and characterizing intracellular  
signaling mechanisms (Ross et al., *Nature* 294:654 (1981),  
Kozma et al., *Method. Enzymol.* 201:28 (1991), White and  
Backer, *Method. Enzymol.* 201:65 (1991), Kamps, *Method.*  
10 *Enzymol.* 201:101 (1991)). Their value derives from two  
properties; 1) their ability to discriminate whether or not a  
protein is tyrosine phosphorylated, and 2) their ability to react  
with a large variety of different proteins. These properties  
have proven invaluable in tracing intracellular signaling pathways  
and identifying new targets of activated tyrosine kinases.

15  
Ideally, the most useful phosphotyrosine antibodies should  
be as general as possible, that is they should recognize  
phosphotyrosine independently of the protein sequences in which  
it is embedded (context independent) so as to allow detection of  
20 all possible phosphotyrosine residues. The most successful  
approaches for producing phosphotyrosine antibodies have  
utilized phosphotyrosine or phosphotyramine coupled via their  
free amino groups to keyhole limpet hemocyanin using hetero- or  
bifunctional crosslinking agents (Frackelton et al., *Method.*  
25 *Enzymol.* 201:79 (1991), White and Backer *supra* (1991), Wang,  
*Method. Enzymol.* 201:53 (1991), Kamps *supra* (1991)).  
Although currently produced polyclonal and monoclonal

phosphotyrosine antibodies do recognize many different proteins, they often show crossreactivity with other phosphate containing compounds, for example, mononucleotides (Frackelton et al. *supra* (1991), Kamps *supra* (1991)). More importantly,

5 most phosphotyrosine antibodies raised in this fashion display variable sequence reactivity, depending not only on the phosphorylated amino acid, but also upon the amino acid sequences surrounding phosphotyrosine. For example, the present inventors have observed that most phosphotyrosine

10 antibodies do not recognize phosphotyrosine preceded by proline as found in the activation loop of JNK and hence do not react significantly with activated (tyrosine phosphorylated) JNK [(Tan et al. unpublished observations)]. The reason for the variable reactivity is likely due to the fact that the phosphotyrosine

15 antigen is not presented directly to the immune system in the context of variable surrounding amino acids, but is instead presented as a hapten, inappropriately coupled to the KLH carrier via artificial linkages. This approach tends to produce antibodies that react well with phosphotyrosine but are

20 sometimes blocked by surrounding amino acids as they are not present in the antigen.

Other approaches have utilized total cellular phosphotyrosine containing proteins as immungens (Glenney,

25 *Method. Enzymol.* 201:92 (1991), Wang *supra* (1991)) with considerable success but the context-dependence of the resulting antibody specificities was not carefully determined,

although antibodies raised in this fashion did react with a majority of tyrosine phosphorylated proteins. Estimates as to the fraction of tyrosine phosphorylated proteins detected range from 50% to 94% (Kamps *supra* (1991)).

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Attempts to use the above mentioned techniques to produce similar antibodies for phosphoserine and phosphothreonine have met with limited success. Antibodies produced to date have limited crossreactivity and lower affinity for phosphoserine or phosphothreonine probably due to the poor immunogenicity of these phospho-amino acids compared to phosphotyrosine (Heffetz et al., *Method. Enzymol.* 201:44 (1991)). Context-dependence and low affinity have limited the utility of currently available phosphoserine and phosphothreonine antibodies, especially when compared to phosphotyrosine antibodies

Site-specific phosphoserine and phosphothreonine antibodies were first described by Nairn et al. in 1982 and have proven to be highly useful tools to study protein phosphorylation (Czernik et al., *Method. Enzymol.* 201:264 (1991), Czernik et al., *Neuroprot.* 6:56-61 (1995)). One drawback of this type of antibody is that a different antibody needs to be produced for each site of interest. Clearly, development of antibodies that detect phosphoserine or phosphothreonine in a context-independent fashion would be desirable for use in tracing serine/threonine kinase cascades and in defining their biological

responses. Likewise, development of context-independent phosphotyrosine antibodies would overcome the limitations of currently available antibodies.

- 5 Motif-specific, context-independent antibodies would also be useful in identifying new targets of 14-3-3 action (i.e., other proteins phosphorylated at this motif) and in characterizing the protein kinases that phosphorylate these sites. Likewise antibodies reactive against acetylated lysine would serve as
- 10 useful tools to study the functional significance of acetylation of histones.

- Such antibodies can further be used as general reagents for detecting phosphorylation or other enzymatic modification *in vitro*, such as in high throughput kinase assays for drug
- 15 screens, as a single antibody can be used to recognize many different phosphorylated substrates. Phosphotyrosine antibodies are currently employed in high throughput kinase assays to screen for selective, high affinity tyrosine kinase
- 20 inhibitors. Compounds or drugs that block enzyme activity are detected by their ability to inhibit kinase activity as determined by a reduction of phosphotyrosine antibody binding to phosphorylated substrate. Similar assays can be set up to screen for pharmaceutically useful compounds using antibodies
- 25 produced as described above for phosphoserine, phosphothreonine, or antibodies detecting other protein modifications.

Antibodies that detect short motifs in a context-independent fashion will also be particularly useful in profiling genome wide changes in protein levels and protein modification.

- 5 For example, the use of context-independent phosphothreonine antibodies and 2D gel electrophoresis to profile genome wide changes in protein phosphorylation (Patterson and Garrels, Cell Biology: A Laboratory Handbook 249-257 (1994), Academic Press) as the result of drug treatment or overexpression of a
- 10 particular protein will undoubtedly prove useful in identifying potential drug-protein interactions and suggest new downstream targets for overexpressed proteins.

### SUMMARY OF THE INVENTION

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- In accordance with the present invention, there is provided a method of producing antibodies that selectively recognize specified short amino acid motifs independent of the surrounding amino acid, peptide, or protein sequences. The
- 20 method allows the production of antibodies that recognize modified single amino acids, for example phosphorylated serine, threonine, and tyrosine, or acetylated lysine, as well other unmodified or modified motifs of one or more amino acids.

- 25 The method encompasses the production and purification of highly context-independent antibodies that recognize specific and highly degenerate amino acid motifs, such as those found in

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kinase consensus sequences or other enzyme binding sites. Furthermore, the method can be used to produce highly context-independent polyclonal or monoclonal antibodies.

5           Antibodies produced by the method of the present invention may be specific to virtually any protein motif, either modified or unmodified. For example, the method can be used to produce antibodies recognizing phosphothreonine alone or phosphothreonine in the context of several fixed amino acids as  
10   found in the MAPK, 14-3-3, or cdk consensus sites. It can also be used to produce antibodies specific for other modified amino acids, for example, acetylated lysine, or to detect any short motif of one or more amino acids, in a context-independent fashion.

15           The present invention further provides a method of profiling large and diverse protein populations on a genome-wide scale by utilizing motif-specific, context-independent antibodies against motifs conserved on such proteins. For example,  
20   phosphorylation-specific antibodies allow genome-wide profiling of changes in protein phosphorylation as a result of drug treatment.

          The present invention also provides a method of  
25   identifying an unknown substrate of a known enzyme through the use of motif-specific, context-independent antibodies which

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are raised against motifs common to other substrates of the enzyme.

The use of such motif-specific, context-independent  
5 antibodies as a reagent for the detection of enzymatic  
modifications of a given motif within a substrate is also  
encompassed by the present invention.

### BRIEF DESCRIPTION OF DRAWINGS

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Figure 1a is a table depicting the specificity of the affinity-purified, polyclonal antibodies produced against a phosphorylated threonine peptide library in Example I, when tested against specific peptides.

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Figure 1b is a table depicting the specificity of the phosphothreonine antibodies of Example I when tested against various phosphopeptide libraries.

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Figure 1c is a Western analysis which depicts the reactivity of the phosphothreonine antibodies of Example I against cell extracts from cells treated with and without okadaic acid and against other phosphoproteins.

25

Figure 1d is a table depicting the context-independence of the anti-phosphothreonine antibodies of Example I as shown by immobilized grid.

Figure 2a is a table depicting the specificity of the affinity-purified, polyclonal antibodies produced against a phosphorylated PXS\*P peptide library in Example II.

5

Figure 2b is a Western analysis depicting the reactivity of the phospho-PXS\*P antibodies of Example II against cell extracts from cells treated with and without okadaic acid and against other phosphoproteins.

10

Figure 3a is a table depicting the lack of reactivity of the affinity-purified, polyclonal 14-3-3 antibodies of Example III when tested against non-phosphopeptides or phosphopeptides lacking the motif.

15

Figure 3b is a Western analysis depicting the reactivity of the phospho-14-3-3 antibodies of Example III against cell extracts from cells transfected with GST-Bad and with TPA.

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Figure 4a is a table depicting the specificity of the monoclonal antibodies produced against a phosphorylated PXT\*PXR library in Example IV.

25

Figure 4b is a Western analysis depicting the reactivity of the CDK consensus site monoclonal antibodies of Example IV against phosphorylated and nonphosphorylated RB protein.

Figure 5a is a Western analysis depicting the specificity of the acetylated-lysine antibodies of Example V against acetylated BSA.

5        Figure 5b is a Western analysis depicting the reactivity of the acetylated-lysine antibodies of Example V against various proteins present in C6-cell extracts when antibodies are preincubated with nonacetylated peptide library.

10       Figure 5c is a Western analysis depicting the reactivity of the acetylated-lysine antibodies of Example V against various proteins present in C6-cell extracts when antibodies are preincubated with acetylated peptide library.

15       Figure 5d is a Western analysis depicting the reactivity of the acetylated-lysine antibodies of Example V against the control acetylated BSA when antibodies are preincubated with acetylated peptide library.

20       Figure 6 shows the signal to noise ratio of ELISA readings using phospho-Akt substrate antibody with phospho-peptides of Akt substrates vs. non-phospho-peptides of Akt substrates.

25       Figure 7 is a Western analysis of calyculin A-treated A431 cells using phospho-Akt substrate antibody.

Figure 8 shows the signal to noise ratio of ELISA reading using phospho-PKA substrates antibody against peptides have arginine or lysine at -3 position.

5 Figure 9 is a Western analysis of calyculin A-treated A431 cells using phospho-PKA substrates antibody.

Figure 10 is a Western analysis of A431 cell extracts phosphorylated by protein kinase A, ERK2 and CDC2/cyclinA *in vitro* using phospho-PKA substrate antibody.

Figure 11 shows the signal to noise ratio of ELISA reading using phospho-serine/threonine phenylalanine antibody against the peptides containing phenylalanine, tyrosine or tryptophan.

Figure 12 is a Western analysis of calyculin A-treated A431 cells using phospho-serinine/phenylalanine substrates antibody.

20 DETAILED DESCRIPTION

The present invention is based upon the concept that the concentration of any individual sequence in a peptide library used as antigen is extremely low and hence will be insufficient to drive an immune response in a host. The only antigenic determinants of sufficiently high concentration to drive the immune response

are thus the fixed residues common to each sequence, as well as the peptide backbone itself.

Immunizing a host with peptide libraries representing all 20 amino acids at each degenerate position will produce antibodies tolerant to many, or all, amino acids at the variable positions surrounding one or more fixed residues. Such antibodies will then react with the antigenic determinant in the context of the broadest possible range of surrounding amino acid, peptide, or protein sequences. The fixed residue(s) of the motif may be a single unmodified or modified amino acid, such as a phosphorylated or unphosphorylated residue, or may be multiple unmodified or modified amino acids, such as a consensus recognition site.

As used herein, "antibodies" means polyclonal or monoclonal antibodies, including Fc fragments, Fab fragments, chimeric antibodies, or other antigen-specific antibody fragments.

As used herein, "motif-specific, context-independent antibodies" means antibodies which are specific against one or more fixed amino acid residues in the context of variable surrounding peptide or protein sequences; such antibody specificity is thus highly independent of the context in which the antigen occurs.

As used herein, "substrate" means any target molecule, including peptides or proteins, which an enzyme specifically recognizes and acts upon.

- 5           The general method by which motif-specific, context-independent antibodies are produced in accordance with the present invention is as follows:

- 10           (1) Motif-specific antibodies that react with any protein or peptide containing specific target residues independently of the surrounding amino acids may be obtained by synthesizing a highly degenerate peptide library. In one preferred embodiment, the library comprises XXXXXXJ\*XXXXXXC where X= all 20 amino acids except cysteine and J\*= a modified (\*) amino acid (J), for  
15           example, phosphothreonine (T\*) or acetylated-lysine (K\*). It will be appreciated that the specific target residue may be unmodified and that a shorter or longer library may be generated and less than all of the surrounding amino acids may be varied. In one preferred embodiment, the peptide library is  
20           about 6 to 14 residues long. While the preferred embodiment utilizes one fixed amino acid (either modified or unmodified) in a varied surrounding context, other preferred embodiments may utilize a motif comprising several fixed amino acids. Likewise, the surrounding sequence of the library may be varied at more  
25           than one position simultaneously, or, as in the preferred embodiment, varied at only one surrounding sequence position per degenerate molecule, such that a library is produced which is

completely degenerate at every position except the fixed residue(s). The peptide library can be synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and using mixtures of each amino acid during  
5 degenerate coupling reactions.

The incorporation of modified amino acids at fixed positions should not be limited to phosphorylation or acetylations as other modified protected amino acids can also be  
10 incorporated, for example, amino acids modified with lipids (e.g. farnesylated, isoprenylated) or protected O-linked or N-linked sugars (e.g. glycosylated), methylated, or ribosylated amino acids, or nucleotides, polymers of nucleotides, nucleosides, or amino acids such as ubiquitin, or amino acid analogues.

15

The incorporation of unmodified amino acids at fixed positions may be selected to mimic conserved motifs, for example zinc fingers or repeating arginine residues.

20 (2) In order to produce as equal a representation of each amino acid as possible at each degenerate position, several rounds of altering the amino acid composition, synthesizing, and peptide sequencing are conducted. Amino acid sequence analysis at several different positions along the peptide is conducted to  
25 verify a random amino acid representation at each position and that the random representation is maintained throughout the synthesis. It will be recognized by one of skill in the art that the



number of rounds may vary in order to achieve an equal distribution of all amino acids at each position.

(3) The highly diverse peptide library is used as an antigen, preferably by covalent coupling to a carrier. In a preferred embodiment, keyhole limpet hemocyanin (KLH) emulsified in Freund's adjuvant is used as the coupling agent, and the coupled peptide library injected intradermally into a host, such as female New Zealand white rabbits. Booster injections may be given in incomplete Freund's adjuvant until an immune response is obtained. Antibody titre is measured by a suitable method, such as ELISA against the motif-specific peptide libraries. Antisera raised in this manner may be used in both crude or purified preparations, as outlined below.

(4) Antisera from the most promising hosts are purified, for example over protein A, and adsorbed over a J (non-modified) peptide library column. In the preferred embodiment, the nonadsorbed fraction (flow through) is then applied to a J\* column, eluted at suitable pH, dialyzed and tested for J\* specificity by a suitable method, such as ELISA using J\* and J as antigen.

(5) Antibodies affinity purified in this fashion recognize the J\* peptide library but do not react with the J library and exhibit a high degree of specificity for J\*. These antibodies may be further tested for lack of reactivity against the unmodified

form of the target modified amino acid, J\*, or a J\* homologue, utilizing a suitable method, such as ELISA.

5 (6) Antibodies may be further tested by western blotting, or another suitable method, using cell extracts prepared from cells treated with and without a selected protein modification enzyme inhibitor, such as protein phosphatase inhibitor okadaic acid. Treatments that increase protein modification will increase the number of antibody reactive  
10 proteins as well as the intensity of reactivity. The J\* specific antibodies will react with a relatively small number of proteins from control extracts but will react with a very large number following treatment with the selected inhibitor. The antibodies will show no reactivity with the inactive-non-modified versions of  
15 these proteins, demonstrating a high degree of J\* specificity and suggesting broad cross-reactivity to many different modified -target containing proteins.

20 (7) The degree of context-independence may be more carefully examined, for example, by ELISA analysis against individual J\* peptides that are mixed together or tested individually. Such analysis can indicate if poor reactivity occurs with certain motifs, such as when J\* is followed by proline, for example.

25

(8) The context-dependence of J\* antibody recognition may be further examined, as in the preferred embodiment, using

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a immobilized grid of modified-peptide libraries. In addition to a fixed target residue, J\*, each different library is synthesized to contain an additional fixed amino acid at different positions relative to J\* but with all other positions containing all 20 amino acids except cysteine. Each peptide library is coated, for example, on the bottom of an ELISA well and exposed to the J\* antibodies. Antibodies that do not react with a particular spot (peptide library) on the grid do not bind when the specified amino acid is present at the specified position. This analysis determines whether or not a particular amino acid at a particular position relative to J\* will allow or block binding.

Alternatively, purified antibodies can be linked to beads, allowed to bind the modified or unmodified library, unbound sequences washed away, and bound sequences recovered and subject to amino acid sequencing to determine the amount of each amino acid present at each position in the library. This information will indicate what amino acids are tolerated at each position.

(9) Monoclonal antibodies may be prepared, as in one form of the preferred embodiment, by coupling the J\* peptide library to a suitable carrier, such as KLH, and injected into a host, such as BalbC mice. The J\* peptide-KLH conjugate may be emulsified in Freund's adjuvant and booster injections in incomplete Freund's adjuvant may be carried out every other week until a response is obtained.

(10) Antibody titre is measured by a suitable method, such as ELISA against J\* and non-J\* peptide libraries. Sera from hosts showing high-titre responses are adsorbed with  
5 immobilized non-J\* peptide and the nonadsorbed fraction tested by, for example, western blotting.

(11) Spleens from hosts showing J\*-specific responses are fused to myeloma cells and hybridoma clones are selected  
10 and screened. Supernatants from individual clones are screened first for their ability to bind the J\*-peptide library. Positive clones are next screened for their cross-reactivity against the non-J\* library. Clones showing the highest degree of J\*-  
15 specificity are chosen for further analysis as described above in steps (5) through (8).

(12) Overproduction of monoclonal antibodies resulting from step (11) above may be carried out, for example, by harvesting ascites, culturing selected hybridoma clones, or  
20 cloning into a host organism, such as *E. coli*.

The motif-specific, context-independent antibodies produced by this method may be used to identify an unknown substrate of an enzyme. Such antibodies are first generated  
25 against a motif that is recognized by the enzyme of interest; for example, a consensus site. These antibodies are then used to screen a sample for the presence of other, unknown

substrates which contain the same motif. This method enables the rapid detection of important new substrates in a variety of cascades which involve conserved substrate motifs. For example, antibodies that selectively recognize a wide variety of proteins only when phosphorylated at the MAPK consensus phosphorylation site would greatly facilitate the detection of new MAP kinase targets. MAP kinase could be overexpressed in cell culture, activated by growth factors, and target substrate proteins identified by western blotting using antibodies that selectively recognize the phosphorylated substrate proteins (Stukenberg et al., *Curr. Biol.* 7:338-348 (1997). Alternatively, MAPK could be used to phosphorylate cDNA expression libraries *in vitro* and MAPK consensus-site antibodies used to identify cDNA clones expressing MAPK phosphorylated substrates (Funkunaga and Hunter, *EMBO* 16(8):1921-1933 (1997).

Similarly, antibodies produced by the method of the instant invention may be used to identify an enzyme which modifies a known substrate motif. Such antibodies, whether specific for modified (e.g. phosphorylated) or unmodified (e.g. zinc finger) motifs, can be used to detect whether a certain enzyme of interest has modified a substrate which contains that motif. This method allows for the rapid detection of important new proteins which act on known classes of substrates containing contain conserved motifs, for the example MAPK consensus site.

The motif-specific, context-independent antibodies of the instant invention may also be used *in vitro* as reagents in high-throughput assays, such as drug screens, to detect the enzymatic modification of certain substrates containing a conserved motif. For example, antibodies specific for a certain phosphorylated motif enable the rapid detection of inhibitors of the enzyme that act at that motif. In the case of a drug screen, a single motif-specific antibody can be used to assay the activity of a wide range of enzymes acting at many diverse sequence motifs. Phosphotyrosine antibodies are currently employed in high throughput kinase assays to screen for selective, high affinity tyrosine kinase inhibitors. Compounds or drugs that block enzyme activity are detected by their ability to inhibit kinase activity as determined by a reduction of phosphotyrosine antibody binding to phosphorylated substrate. Similar assays can be set up to screen for pharmaceutically useful compounds using antibodies produced as described above for phosphoserine, phosphothreonine, or antibodies detecting other protein modifications.

Antibody based detection of protein kinase activity has several advantages over radioactive assays for use in automated high throughput kinase assays. First, radioactive assays are difficult to automate because they employ transfer of  $^{32}\text{P}$  gamma-labeled ATP to a peptide substrate. The phosphopeptide is then separated from labeled ATP using phosphocellulose filters and several washing steps, and finally,

phosphorylation is quantitated by liquid scintillation methods. Together these steps are time consuming and difficult to automate. Antibody detection allows a wide variety of ELISA-type assays that are well suited for automation and high throughput screens.

Second, radioactive assays require low levels of ATP to insure high levels of  $^{32}\text{P}$  incorporation for maximal sensitivity. Low levels of ATP in the kinase assay bias the search for inhibitors towards compounds that compete with ATP binding in the protein kinase catalytic cleft. Such screens consistently yield competitive inhibitors at the ATP binding site which due to the highly conserved nature of this binding site results in inhibitors with poor selectivity.

Current high-throughput kinase assays typically utilize biotinylated peptide substrates immobilized on the bottom of a 96 or 386 well plate that is subsequently incubated together with the desired protein kinase, ATP, and the appropriate kinase buffer. Kinase activity is measured using a fluorescently labeled phosphospecific-antibody that reacts only with the phosphorylated peptide substrate. These assays come in two formats homogeneous (not involving wash steps and heterogeneous (involving wash steps). Homogeneous fluorescent assays typically utilize lanthanide-labelled phosphoantibody binding to a phosphorylated peptide substrate that has linked to it an energy acceptor, for example

allophycocyanin. Binding of the phosphoantibody the phosphorylated peptide substrate brings the two fluorophores close enough together to allow fluorescence resonance energy transfer to occur shifting the frequency of the emitted signal, indicating the presence of a biomolecular complex. Different compounds are added to each well and the ability of the compound to inhibit substrate phosphorylation is determined by inhibition of fluorescence energy transfer. This format is similar to the scintillation proximity assay commonly used in radioactive assays. Other homogeneous assays involve the use of fluorescence polarization to measure the binding of phosphoantibody to phosphorylated substrate.

The key feature in the homogeneous assays are the limited number of steps and the ease in automation. A large variety of heterogeneous kinase assays based upon ELISA formats are also currently in use. These assays typically utilizing fluorescently labeled phosphoantibodies binding phosphorylated peptide substrates that are immobilized in 96 or 386 well formats. In this case wash steps are required to separate bound from unbound antibody. Fluorescently labeled antibody retained in the well is then detected using time resolved fluorescence.

The motifs used to generate antibodies for such modification screening assays may be either modified or unmodified substrate motifs. Antibodies generated against



unmodified motifs will not bind if the substrate has been subsequently modified by an enzyme. Similarly, antibodies generated against modified motifs can detect increases in modified substrate concentrations owing to enzymatic activity.

5

Similar approaches may be applied to study a variety of other enzymatic modifications, and are not limited to the protein kinase or acetyltransferase activities discussed below. For example, the approach could be used to generate antibodies that recognize many other types of protein modification, including, but not limited to, the addition of sugars, methyl groups, carboxyl groups, the addition of various lipids, or the addition of nucleotides, or polymers of nucleotides, nucleosides, or amino acids such as ubiquitin.

15

Likewise, such motif-specific, context-independent antibodies may be used on a genome-wide scale to simultaneously profile large and diverse protein populations which contain conserved motifs. A specific two or three amino acid binding site, for example consecutive arginine residues, should appear (based upon a random distribution of amino acids) once every 400 or 8000 residues, respectively, (equating to approximately once per protein, or once every 20 proteins, respectively, (assuming the average protein is 400 amino acids)). Thus, an antibody specific for such a motif independent of the context in which it occurs allows for the rapid screening of a great number of proteins.

25

Phosphorylation specific antibodies allow genome wide profiling of changes in phosphorylation of proteins as a result of drug treatment or the overexpression of specific

5 genes/proteins as a result of such treatment. Such antibodies also facilitate the profiling of expression of specific proteins in sequenced genomes.

For example, suppose that a drug is developed which  
10 inhibits the cell-cycle dependent protein kinase cdc2. The drug has been shown to inhibit cdk2 with high affinity, but the specificity of the compound needs to be further tested to examine whether other protein kinases are inhibited and if so, which ones.

15

As an early step in this process cell lines may be treated with the drug and the effects on total cell protein phosphorylation monitored using a panel of motif-specific and general phosphoantibodies to examine the nature of the  
20 phospho-substrates inhibited by the compound or lead drug.

Total protein from cell extracts prepared from control or drug treated cells may be fractionated using, for example, 2-dimensional gels (isoelectric focusing in the first dimension and  
25 standard SDS-polyacrylamide molecular weight fractionation in the second dimension), transferred to nitrocellulose membranes,

and analyzed by western blotting using, in this hypothetical case, kinase consensus site-specific phosphoantibodies.

In this case, global analysis of total cell proteins using a  
5 cdc2 consensus site specific antibody would provide information  
regarding the ability of the drug to block phosphorylation at all  
potential cdc2 site substrates. The pattern of inhibition at  
other non-cdc2 substrates (i.e. the degree of specificity) could  
also be examined using antibodies to different kinase consensus  
10 sites, or using antibodies to phosphotyrosine to determine  
whether the inhibitor also acts to block tyrosine kinases.

Currently, for mammalian cells, the the identity of the  
majority of protein "spots" visualized on 2-D gels are unknown.  
15 However, as all human genes are identified and sequenced and  
the corresponding proteins characterized and "spots" identified,  
analysis by protein profiling in accordance with the present  
invention will become even more powerfully informative. The  
identity of the proteins inhibited will not only confirm the drug  
20 specificity but the identity of additional "nonspecific" proteins  
inhibited will also suggest possible side effects. Identical  
analysis can be carried out in simpler, completely sequenced  
organisms, such as yeast where many of the protein "spots" on  
2-D gels have already been identified.

25

The Examples presented below are only intended as  
specific preferred embodiments of the present invention and are

not intended to limit the scope of the invention except as provided in the claims herein. The present invention encompasses modifications and variations of the methods taught herein which would be obvious to one of ordinary skill in the art.

The references cited above and below are herein incorporated by reference.

10

**EXAMPLE 1****Context-Independent Phosphothreonine Antibodies:**Synthesis of peptide library antigens:

Phospho-specific antibodies that react with any protein containing phosphorylated threonine residues, i.e that bind phosphothreonine independently of the surrounding amino acids, were obtained by synthesizing a highly degenerate peptide library XXXXXXThr\*XXXXXXC where X= all 20 amino acids except cysteine and Thr\*=phosphothreonine.

The phosphothreonine peptide library was synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and using mixtures of each amino acid during degenerate coupling reactions. Degenerate peptides were synthesized using an ABI model 433A peptide synthesizer, using FastMoc chemistry (Fields et al., *Pept. Res.* 4:95-101 (1991), hereby incorporated by reference herein) at a scale of 0.085 mmol. Fmoc/NMP chemistry utilizing HBTU amino acid activation

(Dourtoglou et al., *Synthesis* 1984: 572-574 (1984), Knorr et al., *Tetra. Let.* 30:1927-1930 (1989), Knorr et al., in Peptides 1988 37-129 (1989), Walter de Gruyter & Co, all hereby incorporated by reference herein) was employed for all cycles.

- 5    Preloaded Fmoc-Cys(Trt) HMP (p-hydroxymethylphenoxymethyl) polystyrene resin functionalized at 0.5 mmol/g was used for each degenerate pool of peptides. Peptides were synthesized using single coupling during each cycle, although coupling times were extended at each position containing a phosphorylated
- 10    amino acid. The final Fmoc was removed during synthesis. Utilization of preloaded HMP resin along with final Fmoc group removal yields peptides having both free amino and carboxy termini after cleavage and deprotection.

- 15            In order to produce as equal a representation of each amino acid as possible at each degenerate position several rounds of altering the amino acid composition, synthesizing, and peptide sequencing were conducted. The desired peptide pools were to contain an equimolar mix of 19 amino acids (all standard
- 20    amino acids except Cys) at each "degenerate" site. Because the rate of reactivity of each protected amino acid differs, simply mixing equimolar amounts (each at approximately 5.26% of total) does not result in a population of peptides that is equimolar at each position. In order to maximize degeneracy at
- 25    each residue, peptide synthesis was first done using equimolar "mixes" at each position. Phenylthiocarbamyl-amino acid analysis was performed therefore allowing assessment of

relative amino acid content at each position. Based on amino acid analysis the molar amounts of each amino acid in the "mix" were adjusted to compensate for different reaction rates, in order to ensure equal representation of each amino acid at each degenerate position. Several rounds of peptide synthesis followed by amino acid analysis were necessary to optimize the amino acid mix, which resulted in a totally degenerate peptide.

The optimized amino acid mix arrived at was as follows: G (4.6%); A (5.6%); V (3.3%); L (2.5%); I (4.25%); S (4.4%); T (8.4%); F (2.25%); Y (6.0%); W (6.8%); M (2.9%); P (2.5%); D (5.8%); N (9.5%); E (6.2%); Q (9.4%); K (6.1%); R (6.4%); H (3.5%).

Cleavage of the degenerate peptides from the resin along with removal of side chain protecting groups occurs simultaneously upon treatment with TFA. The cleavage mixture (Perkin Elmer, Emeryville, CA (1995)) consists of the following: 0.75g phenol, 0.125 ml methyl sulfide, 0.25 ml 1,2-ethanedithiol, 0.5 ml milliQ H<sub>2</sub>O, 0.5 ml thioanisol, 10 ml TFA. The entire mixture was added to the peptide resin (approx. 300 mg). The resin was flushed with nitrogen and gently stirred at room temperature for 3 hours. The resin was then filtered allowing the peptide to be precipitated into cold ( 0°C) methyl-t-butyl ether. The ether fraction was centrifuged allowing collection of the precipitate. The peptide precipitate was vacuum dried, analyzed by mass spectroscopy, and HPLC purified.

A sample of the peptide was dissolved in acetonitrile/water (50:50, v/v) and analyzed on a Perceptive Biosystems (Framingham, MA) MALDI-TOF mass spectrometer using 2,4,6-trihydroxyacetophenone plus ammonium citrate as the matrix. As expected, the peptide mixture did not show a homogeneous product. MALDI-TOF analysis demonstrated that the peptide pool was degenerate, showing an average mass and the expected statistically normal curve of peptide mass.

Peptides were purified using a Waters HPLC system consisting of a Lambda-Max Model 481 Multiwavelength detector, 500 series pumps, and Automated gradient controller. A Vydac semi-preparative C18 column was used for reverse-phase purification. A 60 min. linear gradient, 10%-100% B, was used at a flow rate of 2 ml/minute. Buffer A consisted of 0.1% TFA/H<sub>2</sub>O (v/v) while buffer B consisted of 0.1% TFA/60% CH<sub>3</sub>CN/40% H<sub>2</sub>O (v/v/v). Detection was at 214 nm.

Because the peptide pool was degenerate (as demonstrated by mass spectroscopy) HPLC purification was not expected to yield a homogeneous product. Base-line separation of peptide mixtures was not achieved by this method and it was only intended as a crude purification/desalting step. Mass spectroscopy was performed and all fractions whose mass was within the theoretical range were pooled and lyophilized.

- 31 -

Amino acid sequence analysis at several different positions along the peptide indicated a random amino acid representation at each position and that the random representation was maintained throughout the synthesis. The results indicated the production of highly diverse peptide libraries that would serve as suitable antigens.

Production of Rabbit polyclonal antibodies:

All peptides synthesized contained C-terminal cysteine residues allowing conjugation to the carrier protein (KLH) using the heterobifunctional cross-linking reagent m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS). The conjugation procedure used was as described by the manufacturer (Pierce), although the amount of peptide coupled to KLH was increased to 10 mg in order to provide increased material for immunization and boosting of animals. Scale-up required use of a larger desalting column (Bio-Rad 10 DG (Cambridge, MA)) to remove the excess MBS after reaction to N-termini and the  $\epsilon$ -amino group of KLH Lysine residues.

20

The phosphothreonine peptide library was covalently coupled to keyhole limpet hemocyanin (KLH) (250  $\mu$ grams), emulsified in Freund's adjuvant and injected intradermally into female New Zealand white rabbits. Booster injections (200  $\mu$ grams) in incomplete Freund's adjuvant were carried out every other week until a response was obtained. Rabbit sera was screened at three week intervals for the presence of



phosphopeptide specific immunoreactivity by ELISA using both the phosphothreonine and nonphosphothreonine peptide libraries. When the titre of antibody against phosphopeptide reached  $10^5$ , rabbits were put on a production bleed schedule with bleeds collected every two weeks. When 40 ml of high titre serum were obtained, purification of phosphospecific antibodies was initiated, as described below.

Antisera from the most promising rabbit was purified over protein A and passed over a nonphospho Thr/Ser peptide library column. The nonadsorbed fraction (flow through) was applied to a phosphothreonine column, eluted at low pH, dialyzed and tested for phosphospecificity by ELISA using phospho- and nonphosphopeptides. Antibodies affinity-purified in this fashion recognized the phosphorylated threonine peptide library but did not react with the nonphosphothreonine/serine library, indicating a high degree of specificity for phosphothreonine (see Figure 1a). ELISA results also indicated that the antibodies also reacted specifically with a mixture of 18 different phosphothreonine peptides but showed no reactivity with any of the corresponding nonphosphopeptides (Figure 1b). The antibodies also exhibited a strict preference for phosphothreonine, showing no reactivity with a mixture of 38 different phosphoserine peptides (Figure 1b) or peptides containing phosphotyrosine.

We next tested the antibodies by western blotting using cell extracts prepared from cells treated with and without the protein phosphatase inhibitor okadaic acid. As shown in Figure 1c the phosphothreonine antibodies react with a relatively small number of proteins from control extracts but react with a very large number following treatment with okadaic acid (see the smear of high Mol Wt. reactive proteins in Figure 1c, lane 2). The antibodies also reacted specifically with the active forms of MAPK (ERK1) and MKK3 only when phosphorylated at threonine residues at their respective activation loops. The antibodies showed no reactivity with the inactive-nonphosphorylated versions of these proteins (Figure 1c, lanes 3-6). These results demonstrate a high degree of phosphothreonine specificity and suggest broad cross-reactivity to many different threonine-phosphorylated proteins and peptides.

To examine more carefully the degree of context-independence, ELISA analysis was conducted against individual threonine phosphorylated peptides that were mixed together in the previous experiment. As shown in Figure 1a, the phosphothreonine antibody reacts well with all phosphopeptides except those where phosphothreonine is immediately followed by proline, for example the c-Myc and APP1 phosphopeptides (Figure 2b). These results indicate that purified rabbit antibodies reacted in a phosphospecific manner with a wide variety of phosphothreonine but react only poorly with

phosphopeptides where the phosphorylated threonine is followed by proline.

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09535364-032400

The context-dependence of phosphothreonine antibody  
5 recognition was further examined using a immobilized grid of  
phosphopeptide libraries. In addition to a fixed  
phosphothreonine, each different library was synthesized to  
contain an additional fixed amino acid at the -4, -3, -2, -1, +1,  
+2, +3 positions relative to phosphothreonine but with all other  
10 positions containing all 20 amino acids except cysteine. Each  
peptide library was coated on the bottom of an ELISA well and  
exposed to the phosphothreonine antibodies. Antibodies that do  
not react with a particular spot (peptide library) on the grid do  
not bind when the specified amino acid is present at the  
15 specified position. This analysis determines whether or not a  
particular amino acid at a particular position relative to  
phosphothreonine will allow or block binding (Figure 1d).

Subt D2

20 Results confirmed that the phosphothreonine antibodies  
tolerated all amino acids in the -1, -2, -3, -4, and +2, +3 position,  
and bound equally well to every amino acid except proline at the  
+1 position (see Figure 1d, first row). The reactivity as defined  
by this binding profile indicates that the antibodies will bind all  
phosphothreonine containing sequences except those followed  
25 immediately in -1 position by proline. Further analysis using a  
variety of specific phosphothreonine containing peptides  
confirmed these results.

Phosphothreonine specific antibodies from several other rabbits immunized with the same peptide library antigens were further purified and characterized. Antibodies purified from sera obtained from two other rabbits also produced broadly cross-reacting phosphothreonine antibodies as determined by ELISA. One rabbit produced antibodies that react equally well with peptides containing proline following the phosphothreonine. Taken together, these results demonstrate the broad context-independence of the phosphothreonine response obtained when combinatorial peptide libraries are used as immunogens.

## EXAMPLE II

### Protein Kinase Consensus Site-Specific Phosphoantibodies:

#### MAPK-consensus recognition sites: PXS\*P

A peptide library of the preferred site for MAPK phosphorylation PXS\*P was synthesized (Figure 2a) substantially as described in Example I. In addition to an equimolar mix of phosphoserine and threonine, amino acids at two other positions were also fixed; proline at -2 and proline at +1. This library was coupled to KLH and injected into rabbits as described for phosphothreonine. IgG from the most promising rabbit was protein A purified and passed over a nonphospho-Thr/Ser peptide library column. The nonadsorbed fraction (flow through) was applied to a phospho-PXS\*P column, eluted at low pH,

dialyzed and tested for phosphospecificity by ELISA using phospho- and nonphosphopeptides.

Antibodies affinity purified in this fashion reacted strongly  
5 with the phosphorylated PXS\*P peptide library but did not react  
with the nonphosphothreonine/serine library (see Figure 2a).  
ELISA results also indicated that the antibodies also reacted  
specifically with a mixture of 18 different phosphothreonine  
10 peptides but showed no reactivity with any of the corresponding  
nonphosphopeptides (Figure 2a). In addition to being  
phosphospecific, the antibodies exhibited a preference for  
proline at the -2 and +1 positions and showed no reactivity with  
phosphorylated peptides that lack proline at this position (Figure  
2a). The antibodies reacted strongly with the RB and cdk4  
15 phosphopeptides but showed no reactivity with the MKK3,  
PKCalpha, or p70S6 phosphopeptides that lack proline at the +1  
position (Figure 2a). These antibodies do react with some  
peptides lacking proline at -2, for example the cdk4  
phosphopeptide, suggesting that proline at this position is not  
20 absolutely necessary.

PXS\*P antibodies were further tested by western blotting  
using cell extracts prepared from cells treated with and without  
the protein phosphatase inhibitor okadaic acid. Binding of the  
25 PXS\*P antibodies to cell extracts from RS 4;11 cells was  
strongly enhanced following treatment with okadaic acid (smear  
of high Mol Wt. proteins in Figure 2b, lane 2). The antibodies also

reacted specifically with ATF-2 phosphorylated in vitro with MAP kinase but not the nonphosphorylated form of this protein (Figure 2b, lanes 3 and 4), demonstrating a high degree of phospho-specificity and broad cross-reactivity to many different phosphorylated proteins and peptides.

The specificity of PXS\*P antibody recognition was also examined using an immobilized grid of phosphopeptide libraries. As described above, in addition to a fixed phosphothreonine or phosphoserine, each different library was synthesized to contain an additional fixed amino acid at the -1, +1, +2 positions relative to phosphothreonine but with all other positions containing all 20 amino acids except cysteine.

The PXS\*P antibody reacted weakly with peptide libraries where proline was fixed at the -1 position and reacted strongly with libraries where proline was fixed at both the -2 and +1 positions. The reactivity as defined by this binding profile indicates that the PXS\*P antibodies strongly bind only sequences containing the PXS\*P motif, as expected, but that the antisera still contain some residual reactivity to S\*P (as a result of impurities), which could be removed by further purification using immobilized S\*P peptide library.

**EXAMPLE III****Protein Kinase Consensus Site-Specific  
Phosphoantibodies:**5 **14-3-3 binding site: RSXS\*XP**

- Antibodies that identify 14-3-3 targets were obtained by synthesizing a peptide library: XXXXRSXS\*XPXXXXC where S\* is phosphoserine and X represents any amino acid and C is cysteine. The above 14-3-3 phosphopeptide library was
- 10 synthesized by standard F-Moc solid phase peptide synthesis using an ABI peptide synthesizer and mixtures of each amino acid except cysteine during degenerate coupling reactions, as discussed in Example I.
- 15 The 14-3-3 phosphopeptide library was coupled to KLH and injected into rabbits as described above for phosphothreonine and PXS\*P. Antisera from the most promising rabbit was purified over protein A and adsorbed over a nonphospho-14-3-3 peptide library column. The flow-through of this column was
- 20 applied to a phospho-14-3-3 column eluted at low pH, dialyzed and tested for phosphospecificity by ELISA using phospho- and nonphospho-14-3-3 peptide libraries. These affinity purified phospho-14-3-3 antibodies recognized the phosphorylated 14-3-3 peptide library but not the nonphospho-14-3-3 library,
- 25 indicating a high degree of specificity for phospho-14-3-3 (see Figure 3a). The antibodies also reacted strongly with several different peptides containing the 14-3-3 motif including; phospho-Bad-Ser136, cdc25-Ser216, and more weakly with

phospho-Bad-Ser112 which contains a slight variant motif. The antibodies showed no reactivity with the corresponding nonphospho-peptides (Figure 3a) or with many other phosphopeptides that did not contain the motif.

5

Phospho-14-3-3 antibodies were further tested by western blotting using cell extracts prepared from cells transfected with a GST-Bad fusion protein and treated with and without the phorbol ester TPA. The antibodies reacted with a small number of proteins from control extracts (see Figure 3b). Bad was detected in extracts prepared from transfected cells but not control cells. Since the basal level of Bad phosphorylation is high it was difficult to see increased phosphorylation with TPA, although TPA did induce the phosphorylation of several higher molecular weight proteins (arrow in Figure 3b). These results indicate that the phospho-14-3-3 antibodies can detect phosphorylated Bad and other TPA stimulated phospho-proteins.

20 ELISA analysis against the previously described grid of serine/threonine phosphorylated peptide libraries was also conducted. As expected, the phospho-14-3-3 antibodies have an absolute requirement for proline at the +2 position.



**EXAMPLE IV****Production of Mouse Monoclonal Antibodies: CDK  
Consensus Phosphorylation Site PXT\*PXR:**

5

The PXT\*/S\*PXR sequence represents a consensus phosphorylation site for many of the cell cycle-dependent proteins kinases (cdks). Antibodies that recognize this phosphorylated motif would be useful to identify new cdk substrates important in controlling cell cycle progression. The PXT\*/S\*PXR peptide library shown in Figure 4a was coupled to KLH and injected into Balb/c mice. The phosphopeptide-KLH conjugate (50  $\mu$ grams) emulsified in Freund's adjuvant was injected IP. Booster injections (12.5 to 25  $\mu$ grams) in incomplete Freund's adjuvant were carried out every three weeks until a response was obtained. Antibody titre was measured by ELISA against the immunized phosphopeptide library. Sera from mice showing high-titre responses were adsorbed with immobilized nonphospho Thr/Ser peptide and the nonadsorbed fraction tested by western blotting (data not shown).

Splenocytes from a mouse showing phosphospecific responses were fused to myeloma X63Ag8.635 cells (Kearney et al., *J. Immunol.* 123:1548-1550 (1979)) and approximately 1,100 hybridoma clones were selected and screened. Supernatants from individual clones were screened first for their ability to bind the immunized phosphopeptide library and

next for their cross-reactivity against the non-phosphopeptide library. Two different clones showing the highest degree of phospho-specificity were chosen for further analysis. The specificity of clones 6B8 and 5A9 were further characterized using the phosphopeptide libraries and phosphopeptides shown in Figure 4a. Both clones reacted specifically with phosphothreonine containing libraries and individual peptides but did not significantly react with phosphoserine containing peptides, indicating that phosphothreonine selective clones had been identified. Both clones reacted strongly with peptide libraries where proline is fixed in the -2 and +1 positions relative to phosphothreonine. Reactivity against T\*P and PXT\*P libraries does not indicate relaxed specificity since one of 400 and one of 20 peptides in the respective libraries will have the appropriate amino acids at the fixed positions. Both clones reacted strongly with a single RB phosphothreonine peptide containing each of the fixed positions present in the immunized library but did not react significantly with the corresponding nonphosphopeptide.

Western analysis shows that okadaic acid treatment of cultured cells dramatically increases the reactivity with both clones 6B8 and 5A9 (Figure 4b). Clone 6B8 is also shown to detect cdc2 phosphorylated RB by western blotting (Figure 4b) but does not react with nonphosphorylated RB protein. Clone 5A9 was deposited in accordance with the terms and conditions of the Budapest Treaty on September 4, 1998 with the

American Type Culture Collection under ATCC Accession No.  
HB12563.

### EXAMPLE V

5

#### Acetylated Lysine Specific Antibodies:

Antibodies specifically reactive against acetylated lysine  
but not reactive against non-acetylated lysine were obtained by  
10 synthesizing the following acetylated lysine peptide library:  
XXXXXXXXK\*XXXXXXC where K\* is acetylated and X represents any  
amino acid except cysteine and C is cysteine. The acetylated  
lysine peptide library was synthesized as described previously by  
standard F-Moc solid phase peptide synthesis using commercially  
15 available fully protected acetylated lysine.

The peptide library was coupled to KLH and injected into  
rabbits. The K\*-peptide-KLH conjugate (250  $\mu$ grams) was used  
as immunogen as described for the other phosphopeptide  
20 libraries. Antisera from the most promising rabbit were purified  
over protein A and adsorbed over a non-acetylated lysine peptide  
library column. The flow through of this column was applied to  
an acetylated lysine column, eluted at low pH, dialyzed and  
tested for phosphospecificity by ELISA.

25

Acetylated-lysine antibodies, affinity purified as described  
above, recognized the acetylated lysine peptide library but not

the non-acetylated library, indicating a high degree of specificity for acetylated lysine as measured by ELISA. The antibodies also reacted specifically with as little as 0.5 ng of acetylated bovine serum albumin (BSA) but showed no reactivity with up to 10  
5  $\mu$ grams of nonacetylated BSA (see Figure 5a).

The antibodies were further examined by western blotting using cell extracts prepared from cells treated with and without anisomycin. The antibodies react with a number of different  
10 proteins present in the C6-cell extracts (Figure 5b). In panels b and c, antibodies were preincubated with 1  $\mu$ gram of nonacetylated peptide library (Figure 5b) or 1  $\mu$ gram of acetylated peptide library (Figure 5c). Preincubation with nonacetylated peptide library had little effect on antibody  
15 reactivity with acetylated control protein or bands visualized in the cell extract (Figure 5c, lanes 5-8). However, preincubation of the antibodies with the acetylated lysine peptide library completely blocked antibody binding to control acetylated BSA as well as binding to many proteins present in the cell extract  
20 (Figure 5d, lanes 9-12). These results demonstrate a high degree of specificity for acetylated lysine and indicate that the antibodies recognize a broad spectrum of different sized proteins that contain acetylated lysine in a variety of surrounding sequence contexts (compare Figure 5c and d, lanes  
25 1, 2).

**EXAMPLE VI****Phosphoantibody to the Substrate Consensus Sequence  
for Akt: RXRXXT\***

5

The Akt protein kinase is an important regulator of cell survival and insulin signaling, but very few of its *in vivo* targets have been identified. Studies with synthetic peptide substrates of Akt (D.R. Alessi et al. FEBS Lett. 399:333-338 (1996)) as well as the analysis of known Akt phosphorylation sites on GSK-3 (T.F. Franke et al. Cell 88:435-437 (1997)), Bad (M. Pap et al. J. Biol. Chem. 273:19929-19932 (1998); Datta et al. Cell 91:231-241 (1997)), FKHR Brunet et al. Cell 96:857-868 (1999)), and Caspase-9 (M.H. Cardone et al. Science 282:1318-1321 (1998)) indicate that Akt phosphorylates its substrates only at a serine or threonine in a conserved motif characterized by arginine at positions -5 and -3.

20 To study and discover new Akt targets, an antibody was developed that specifically recognizes the phosphorylated form of the Akt substrate consensus sequence RXRXXT\*. This antibody was raised against the following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Thr\* represents phospho-threonine: Cys-X-X-X-Arg-X-Arg-X-X-Thr\*-X-X-X-X. The synthetic phospho-peptide was conjugated KLH (keyhole limpet hemocyanin) and injected into rabbits. Test bleeds were collected

and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography is then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Phospho-Akt Substrate Antibody was found to be already highly phospho-specific as crude serum, so that a subtraction step on a column containing the non-phospho peptide was not necessary and the elution from the Protein A column could be used directly for affinity chromatography on a phospho-peptide-containing column. Protein A eluate was incubated with phospho-peptide resin by rotation in a sealed column at room temperature for one hour. Column was then drained, washed twice with PBS, and eluted with 0.1M Glycine, pH 2.7 and pooled fractions neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody is highly specific for peptides which contain phospho-threonine/serine preceeded by arginine at positions -5 and -3 (Figure 6) Some cross-reactivity is observed for peptides which contain arginine at positions -3 and -2. (Figure

6) also shows that this antibody is highly phospho-specific and recognizes these motifs only when phosphorylated (signal to noise ratios were determined as a ratio of reactivity with the phospho-peptide to reactivity with the corresponding non-phospho-peptide). This antibody does not recognize other phospho-threonine/serine containing motifs. (Figure 7) indicates that in mammalian cells there are many phosphoproteins recognized by this antibody.

## EXAMPLE VII

### **Phosphoantibody to the Substrate Consensus Sequence for PKA: RRXT\***

cAMP-dependent Protein Kinase A (PKA) is an important kinase for regulating a striking number of physiologic processes, including intermediary metabolism, cellular proliferation and neuronal signaling, by altering basic patterns of gene expression (M. Montminy Annual Rev. Biochem. 66:807-822 (1997)). Studies with synthetic peptide substrates have established a consensus phosphorylation site for PKA, namely serine or threonine with arginine at the -2 and -3 positions (Z. Songyang *et al.* Current Biology 4:973-982 (1994)).

To identify and study new *in vivo* substrates of PKA, an antibody was developed that specifically recognizes the phosphorylated form of the PKA substrate consensus sequence RRXT\*. The following synthetic phospho-peptide peptide antigen

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1033  
5  
was used, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Thr\* represents phospho-threonine: Cys-X-X-X-X-X-Arg-Arg-X-Thr\*X-X-X-X. The synthetic phospho-peptide was conjugated KLH (keyhole limpet hemocyanin) and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

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10 Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to  
15 isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Protein A eluate was first  
20 incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. This resin was then drained and the flow-through then incubated with phospho-peptide resin. This column was drained,  
25 washed twice with PBS, phospho-specific antibody eluted with 0.1M Glycine, pH 2.7 and pooled fractions neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.



Figure 8 shows that the resulting antibody is highly specific for peptides or proteins containing phospho-threonine with arginine at the -3 position. The antibody also recognizes  
5 some proteins containing phospho-serine with arginine at the -2 and -3 position. It does not recognize the non-phosphorylated version of these motifs (as shown by the signal to noise ratios in Figure 8 which were determined as a ratio of reactivity with the phospho-peptide to reactivity with the corresponding non-  
10 phospho-peptide); nor does the antibody recognize other phospho-serine/threonine containing motifs. Figure 9 indicates that in mammalian cells there are many phosphoproteins recognized by this antibody, while Figure 10 shows that this antibody specifically detects many PKA protein substrates in a cell but will not  
15 recognize substrates of the ERK2 or CDC2 kinases, which have different substrate specificities.

### EXAMPLE VIII

20 **Phosphoantibody to the Substrate Consensus Sequence for Bulky Ring-Directed Kinases: [F/Y][T/S]\* or [S/T]\*F**

Some important classes of protein kinases are regulated by phosphorylation of a specific serine or threonine flanked by either  
25 phenylalanine or tyrosine. For example, Akt, which plays a central role in regulating cell survival, is activated by phosphorylation at Ser473, a site flanked by phenylalanine and tyrosine (D.R. Alessi

*et al.* EMBO J. 15:6541-6551 (1996)). RSK1 (Ser381) and the PKC's also contain this consensus site, phosphorylation of which is required for their activity (K.N. Dalby *et al.* J. Biol. Chem. 273:1496-1505 (1998); L.M. Keranen *et al.* Curr. Biol. 5:1395-1403 (1995)).

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5/24/94  
10 To help study signaling pathways regulated by phosphorylation at these key regulatory sites we developed an antibody that detects phospho-serine and phospho-threonine only when preceded by tyrosine, tryptophan or phenylalanine or when followed by phenylalanine. This antibody was raised against the following synthetic peptide antigen, where X represents a position in the peptide synthesis where a mixture of all twenty amino acids were used, and Ser\* or Thr\* represents phospho-serine or phospho-threonine: X-X-X-X-F-X-X-F-[S\*/T\*]-[F/Y]-X-X-X-X-C. This synthetic phospho-peptide was conjugated to KLH and injected into rabbits. Test bleeds were collected and characterized by ELISA on phospho and non-phospho versions of the peptide antigen.

20 Once rabbits started to show high phospho-specific titers, 40ml production bleeds were obtained. Bleeds were dialyzed overnight in 0.025M NaAcetate, 0.01M NaCl pH=5.2 at 4°C, then spun at 11,200 rpm at 4°C for 30min to precipitate serum lipids. Serum supernatant was then purified by Protein A  
25 chromatography on a Pharmacia (Piscataway, NJ) ÄKTA FPLC to isolate the IgG antibody fraction. Affinity chromatography was then performed using peptide coupled to SulfoLink resin from

Pierce (#20401; coupling directions according to manufacturer). Both phospho-peptide-containing resin and the corresponding non-phospho-peptide resin were prepared. Two rounds of subtractive purification were performed using the non-phospho-peptide resin:

5 Protein A eluate was incubated with non-phospho-peptide resin by rotation in a sealed column at room temperature for one hour, in order to remove antibodies reactive with the non-phospho version of the protein antigen. The column was drained and the flow-through (containing the desired antibody) incubated with fresh

10 non-phospho-peptide resin. The flow-through from this second subtractive step was finally positively purified by incubation with phospho-peptide resin. After the phospho-peptide column was drained and washed twice with PBS, phospho-specific antibody (bound to the resin) was eluted with 0.1M Glycine, pH 2.7 and

15 pooled fractions were neutralized with 1M Tris-HCl, pH 9.5 (~1-2% of fraction volume). The eluted phospho-specific antibody was then dialyzed overnight in PBS at 4°C.

The resulting antibody is highly specific for

20 phosphorylated [F/Y][T/S]- or [S/T]F- containing peptides (Figure 11). It does not recognize non-phosphorylated [F/Y][T/S] or [S/T]F motifs or other phospho-serine/threonine containing proteins and peptides (signal to noise ratios were determined as a ratio of reactivity with the phospho-peptide to reactivity with the

25 corresponding non-phospho-peptide). This antibody does not recognize other phospho-threonine/serine containing motifs.

- 51 -

Figure 12 indicates that in mammalian cells there are many phosphoproteins recognized by this antibody.

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